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Immunity
Review

Immune Sensing of DNA

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Although it has been appreciated for some years that cytosolic DNA is immune stimulatory, it is only in the past five years that the molecular basis of DNA sensing by the innate immune system has begun to be revealed. In particular it has been described how DNA induces type I interferon, central in antiviral responses and a mediator of autoimmunity. To date more than ten cytosolic receptors of DNA have been proposed, but STING is a key adaptor protein for most DNA-sensing pathways, and we are now beginning to understand the signaling mechanisms for STING. In this review we describe the recent progress in understanding signaling mechanisms activated by DNA and the relevance of DNA sensing to pathogen responses and autoimmunity. We highlight new insights gained into how and why the immune system responds to both pathogen and self DNA and define important questions that now need to be addressed in the field of innate immune activation by DNA.

Introduction

The innate immune system utilizes a limited number of germline-encoded receptors, called pattern recognition receptors (PRRs), to recognize non-self microbial products (pathogen-associated molecular patterns, PAMPs) and host molecules (damage-associated molecular patterns, DAMPs) in order to mount an appropriate immune response to the presence of a pathogen and/or cellular or tissue damage. The first identified—and best characterized—class of PRRs is the Toll-like receptors (TLRs) (Lemaitre et al., 1996; Medzhitov et al., 1997), which are expressed on the cell surface and in endosomal compartments, in order to respond to extracellular and endosomal PAMPs and DAMPs. Cytosolic PRRs that sense microbial and host nucleic acids in the cytoplasm have more recently been discovered (Kato et al., 2011; Keating et al., 2011), and this coupled to the renewed interest in the immune stimulatory properties of DNA in recent years has led to new insights into immune sensing of exogenous and host DNA.

Figure 1 summarizes biological responses to DNA mediated by the innate immune system (Ishii et al., 2006; Stetson and Medzhitov, 2006; Rebsamen et al., 2009; Kaiser et al., 2008; Muruve et al., 2008; McFarlane et al., 2011; Rasmussen et al., 2011; Wenzel et al., 2012; Upton et al., 2010, 2012). Immune sensing of DNA is involved in both early activation of defense against infections (Ishii et al., 2006; Stetson and Medzhitov, 2006) and subsequent bridging to activation of adaptive immune responses (Ishii et al., 2008; Kis-Toth et al., 2011). DNA sensing is also involved in the pathogenesis of some autoinflammatory diseases, most notably systemic lupus erythematosus and Aicardi-Goutières syndrome (AGS) (Leadbetter et al., 2002; Stetson et al., 2008). Since the identification of the first endosomal DNA sensor (Hemmi et al., 2000), the field of DNA sensing has experienced an immense expansion, and we are now beginning to understand the molecular and cellular mechanisms of action of the DNA-sensing machinery. In this review we pay particular attention to how type I interferon (IFN) is induced by DNA and to what is currently known about the role of DNA sensing in host defense, disease, and immunity.

Source and Location of Immunostimulatory DNA

The immunostimulatory activity of exogenously added DNA has been known for 50 years (Isaacs et al., 1963; Rotem et al., 1963). DNA is normally present in the nucleus of eukaryotic cells, and the presence of DNA in aberrant locations, such as the cytoplasm and endosomes, is believed to trigger immune activation (Lund et al., 2003; Ishii et al., 2006; Stetson and Medzhitov, 2006; Kerur et al., 2011). Thus, an early paradigm in the field of innate DNA sensing was that the nucleus is "immune privileged" and that the presence of DNA in other compartments including endosomes and the cytosol activates DNA recognition systems to detect both DNA genomes of invading pathogens (DNA PAMPs) and disturbed self (DNA DAMPs).

It is now well established that unmethylated CpG DNA motifs, which are abundant in many pathogen genomes, have the ability to stimulate immune responses through an endosomal TLR pathway, whereas classical B form double-stranded (ds) DNA is a potent immune stimulator when present in the cytosol (Figure 2; Hemmi et al., 2000; Stetson and Medzhitov, 2006; Ishii et al., 2006). However, recent work has revealed that single-stranded DNAs with specific signatures, including AT-rich stem loop regions (Sharma et al., 2011), also activate immune responses.

The first-described PRR for DNA, and still the only endosomal-based DNA sensor known, was TLR9 (Hemmi et al., 2000), which is expressed preferentially in plasmacytoid dendritic cells (DCs) (Table 1; Kadowaki et al., 2001). TLR9, which is activated by a pathway involving proteolytic cleavage of the ectodomain, recognizes CpG DNA (Ahmad-Nejad et al., 2002; Ewald et al., 2008; Hemmi et al., 2000; Yasuda et al., 2009). Probably the best evidence for TLR9 directly binding DNA is the demonstration of direct interaction between a TLR9-Fc fusion protein and CpG DNA in vitro (Latz et al., 2007). TLR9 is a potent inducer

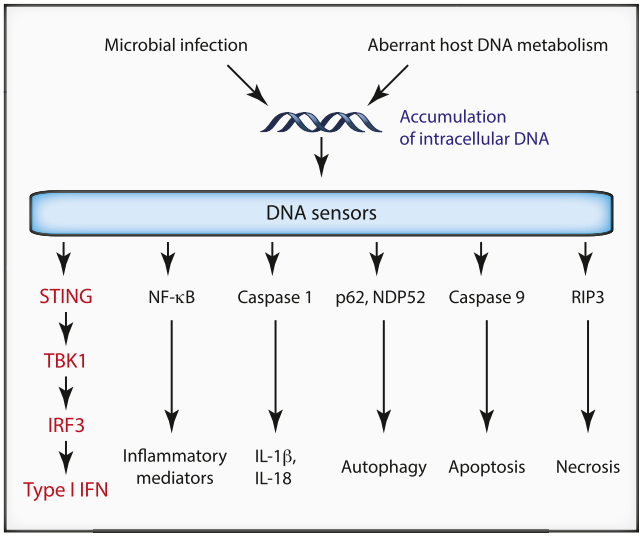

Figure 1. Cellular Functions Stimulated by DNA
Intracellular DNA is recognized by DNA sensors leading to activation of multiple pathways. The best-characterized DNA-stimulated pathway is the one leading to activation of IRFs and induction of IFNs. Other well-characterized pathways activated by DNA recognition are the inflammatory NF-κB and inflammasome pathways, which stimulate expression of inflammatory genes and cleavage of pro-IL-1β and IL-18, respectively. Intracellular DNA also stimulates autophagy and different types of cell death.

of IFN-α expression, which is driven by a signaling pathway dependent on the common TLR adaptor MyD88 and involving IFN regulatory factor 7 (IRF7), which is constitutively expressed in pDCs.

Apart from endosomal sensing of DNA, DNA can end up in the cytosol through several routes as depicted in Figure 2. These include infections with intracellular pathogens, impaired ability to clear exogenous DNA normally metabolized in endo-lysosomes, and imbalanced control of endogenous DNA products and turnover. Cells are equipped with DNases that prevent unwanted accumulation of DNA. DNase II is localized in lysosomes and digests DNA from pathogens and dead cells that end up in this cellular compartment (Okabe et al., 2005). This system is involved in degradation of apoptotic cells taken up by macrophages. In cells devoid of DNase II activity, DNA may leak into the cytoplasm and stimulate cytosolic DNA sensors (Okabe et al., 2005). Another cellular DNase is TREX1 (three prime repair exonuclease 1), which is localized in the cytoplasm and associated with the endoplasmic reticulum (ER). TREX1 is believed to degrade endogenous DNA accumulating in the cytoplasm under homeostatic conditions. It has been reported that DNA species derived from endogenous retroviruses and DNA replication by-products accumulate in the cytoplasm of TREX1-deficient cells and thus stimulate immune responses (Stetson et al., 2008; Yang et al., 2007).

During infections with intracellular DNA-containing microbes, DNA may be released from the microbe to allow DNA sensing. For intracellular bacteria, there is clear evidence that bacterial DNA is found in the cytosol (Manzanillo et al., 2012; Fernandes-Alnemri et al., 2010). Recently, it was reported that *Mycobacterium tuberculosis* actively secretes its DNA into the cytoplasm of host cells for IFN induction, indicating beneficial consequences of DNA sensing for the bacterium (Manzanillo et al., 2012). In fact, immune escape may be a general paradigm for bacterial DNA activation of innate immunity because type I IFN induction can contribute to bacterial pathogenesis (Monroe et al., 2010). Parasite DNA can also stimulate innate immune responses through intracellular pathways, but the mechanisms of exposure of parasite DNA to the cytosol is undescribed (Sharma et al., 2011).

For DNA viruses it has recently been reported that the herpes simplex virus (HSV) capsid becomes ubiquitinated in the cytoplasm and degraded by the proteasome, which leads to release of DNA into the cytoplasm (Horan et al., 2013). This mechanism was found to be operative in macrophages (Horan et al., 2013). Likewise, adenovirus capsids also get ubiquitinated and degraded by a proteasomal pathway (Yan et al., 2002). Thus, specific targeting of viral capsids for proteasomal degradation could be a general mechanism for release of viral DNA into the cytoplasm for immune detection. Because many DNA viruses replicate in the nucleus, it is probably essential for them to be able to bypass capsid sensing and degradation in the cytoplasm and hence exposure of DNA for innate sensors.

Discovery of Signaling Pathways Activated in Response to DNA

Much has been learned recently about the immune signal transduction pathways mediating type I IFN induction in response to cytosolic dsDNA, beginning with the definition of the key kinase complex and transcription factor activated by cytosolic DNA, namely TANK-binding kinase 1 (TBK1) and IRF3, respectively (Stetson and Medzhitov, 2006; Ishii et al., 2006). The search for upstream sensors of cytosolic DNA stimulating the TBK1-IRF3 axis first led to identification of DAI (also known as ZBP1) (Takaoka et al., 2007). DAI was shown to colocalize, and to interact in vitro with, dsDNA and reduction of HSV-1-induced IFN-β expression in the murine fibroblast cell line L929 was observed after DAI knockdown (Takaoka et al., 2007). Subsequent work has confirmed a role for DAI in induction of the type I IFN response during cytomegalovirus (CMV) infection in human foreskin fibroblasts (DeFilippis et al., 2010). In contrast to this, it has been difficult to find essential roles for DAI in DNA sensing in leukocytes (Unterholzner et al., 2010; Ishii et al., 2008) or in vivo (Ishii et al., 2008). Importantly, it has been reported that DAI stimulates necrosis in fibroblasts during MCMV infection through a RIP3-dependent pathway and this activates an antiviral response (Upton et al., 2010, 2012). Thus, the function of DAI in DNA-driven innate immune responses may be cell type dependent. RNA polymerase III (Pol III) was the second cytosolic DNA sensor discovered and has been reported to use AT-rich and herpesvirus DNA as a template to produce 5′ triphosphate RNAs that induce type I IFN through the RNA PRR RIG-I (Ablasser et al., 2009; Chiu et al., 2009). However, the role and physiological implications of Pol III in innate DNA sensing remains to be understood in detail. Furthermore, Pol III could not account for DAI-independent sensing of non-AT-rich DNA, so it was clear that further cytosolic detection systems for DNA exist.

Crucially, a new adaptor protein called STING (also called MPYS, MITA, and ERIS) was discovered as having a central role in responding to DNA by mediating TBK1-dependent IRF3 activation in response to the presence of cytosolic dsDNA

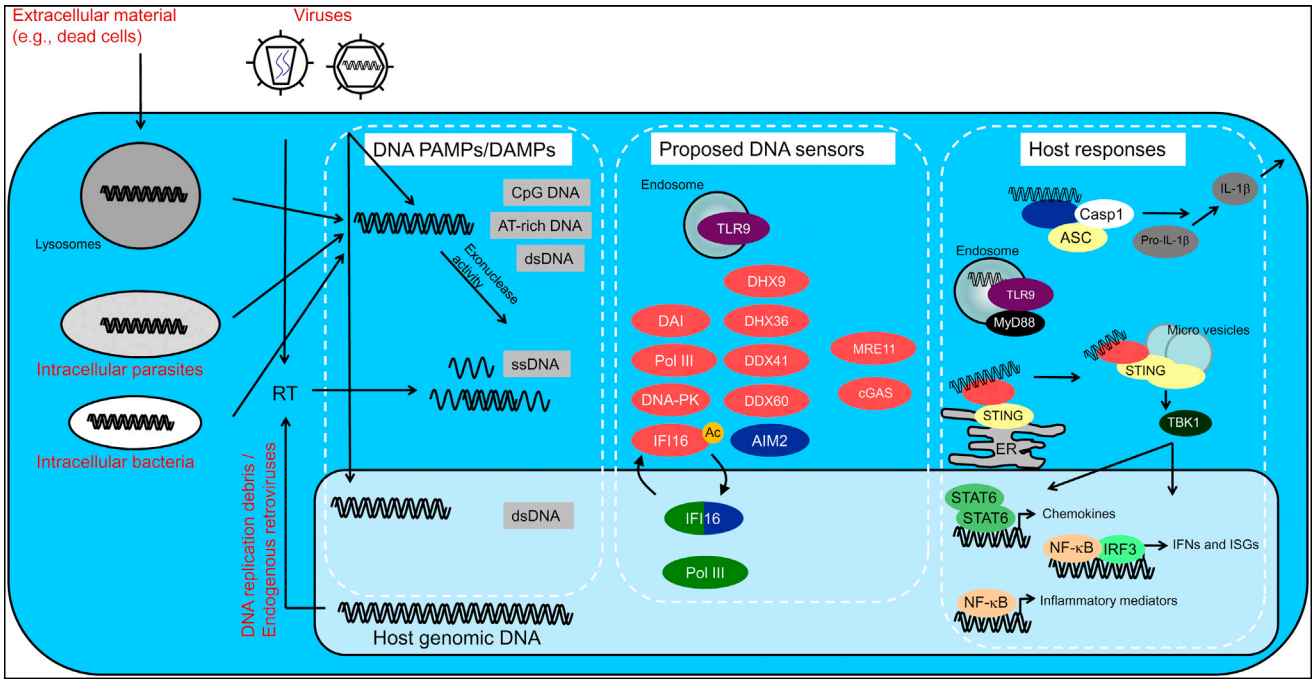

Figure 2. Immunostimulatory DNA and Host Sensors

DNA from microbes or the host has the potential to activate innate immune responses if delivered to the cytoplasm and in some instances also the nucleus. DNA can end up in the cytoplasm through a variety of different pathways, and several different proteins have been proposed to function as PRR for DNA. The main DNA-activated signaling pathways proceed through MyD88 for TLR9, DHX9, or DHX36, through ASC-caspase1 for AIM2, and via STING-TBK1-IRF3 for most other cytosolic IFN-inducing sensors. Colors are as follows: purple, DNA sensors stimulating IFN expression upon DNA recognition in endosomes; red, DNA sensors stimulating IFN expression upon DNA recognition in the cytoplasm; green, DNA sensors potentially stimulating IFN expression upon DNA recognition in the nucleus; blue, DNA sensors stimulating inflammasome activation.

(Ishikawa and Barber, 2008; Zhong et al., 2008; Jin et al., 2008; Sun et al., 2009). Recently there has been a wealth of information on the role of STING in DNA sensing and on the mechanisms whereby it contributes to signal transduction to IFN induction.

STING Is a Central Adaptor Protein for Cytosolic DNA Sensing

Repeated genetic ablation and biochemical studies have clearly demonstrated that STING takes center stage in intracellular signaling in response to cytosolic DNA (reviewed in Burdette and Vance, 2013). Furthermore, studies with STING knockout mice have shown an essential role for STING in responses to bacterial, viral, and eukaryotic pathogens, to self DNA in the context of autoimmunity, and in the adjuvant effects of DNA in enhancing adaptive immune responses (Burdette and Vance, 2013). The STING protein consists of distinct N- and C-terminal domains: the N-terminal 130 amino acids contain four transmembrane domains that anchor STING in the ER, and the remaining 250 amino acids comprise a carboxy-terminal domain (CTD) assumed to be cytosolic. How STING is "activated" by upstream DNA-sensing events remains an open question (see below and Figure 3). By contrast, there is now a clear mechanism to explain how STING engages with TBK1 to cause IRF3 activation. As such, a STING-TBK1-IRF3 signaling axis is now known to direct type I IFN induction by cytosolic DNA in most cases. Tanaka and Chen (2012) showed that in response to cytosolic dsDNA, the C-terminal tail (CTT) of the CTD of STING provides a scaffold to assemble IRF3 in close proximity to TBK1, leading to TBK1-dependent phosphorylation of IRF3. Thus, STING directs TBK1 to activate IRF3 for DNA-sensing pathways. Previous colocalization studies demonstrated that the STING-TBK1 association occurs in discrete yet-to-be-defined punctate foci in the perinuclear region in the cytosol, whereas inactive STING resides in the ER (Ishikawa et al., 2009). So although the movement of STING from the ER (or associated membranes) to the specific foci in the cytosol correlates with activation of the STING-TBK1-IRF3 signaling axis, what upstream signaling events causes the STING movement is a subject of current active research.

One line of research has shown that STING actually directly recognizes bacterial second messenger molecules called cyclic dinucleotides (CDNs), such as cyclic di-GMP (c-di-GMP) (Burdette et al., 2011). Consistent with this, CDNs are known to stimulate a very similar gene induction profile as cytosolic DNA (McWhirter et al., 2009). Therefore, CDNs can be thought of either as novel bacterial PAMPs detected by STING or indeed as an immune escape strategy by bacteria to activate the STING-TBK1-IRF3-IFN pathway for the benefit of the pathogen. Five research groups have now solved the structure of the STING CTD alone or associated with CDNs (Yin et al., 2012; Ouyang et al., 2012; Huang et al., 2012; Shu et al., 2012; Shang et al., 2012). These structures together demonstrate that the STING CTD forms a dimer in solution when not bound by CDNs and that CDN binding does not stimulate any obvious conformational change of the CTD. This observation, together with the fact that

Table 1. Proposed DNA Sensors

DNA Sensor	Cell Types Examined	Site of DNA Sensing	Response	Evidence for DNA Binding	References
TLR9	pDCs	endosomes	type I IFN	interaction between TLR9-Fc fusion protein and CpG DNA in α-screen	Hemmi et al., 2000; Latz et al., 2004, 2007
DAI/ZBP1	fibroblasts	cytoplasm	IFN-β; necrosis	FRET between B-DNA and DAI; pull-down of DAI ± B-DNA competition	Takaoka et al., 2007; Upton et al., 2012
AIM2	macrophages, DCs	cytoplasm	IL-1β, IL-18	affinity purification of Myc-tagged AIM2 with dsDNA-coupled beads; interaction between rAIM2 and dsDNA in α-screen	Hornung et al., 2009; Fernandes-Alnemri et al., 2009; Bürckstümmer et al., 2009; Roberts et al., 2009
IFI16/p204	macrophages, endothelial cells	cytoplasm, nucleus	IFN-β, CXCL10, IL-6, IL-1β	coprecipitation of cytosolic IFI16 with dsDNA-coupled beads; interaction between rIFI16 and dsDNA in α-screen	Unterholzner et al., 2010; Horan et al., 2013; Kerur et al., 2011
RNA Pol III	EBV^+ B cell, macrophage cell line	cytoplasm, nucleus?	IFN-β	production of IFN-inducing RNA transcripts sensitive to Pol III inhibition; purified core RNA Pol III complex produced IFN-inducing RNAs	Ablasser et al., 2009; Chiu et al., 2009
DNA-PK	293T, MEFs	cytoplasm	IFN-λ1, IFN-β, IL-6	coprecipitation of cytosolic Ku70, Ku80, and DNA-PKcs with dsDNA-coupled beads	Zhang et al., 2011a; Ferguson et al., 2012
DHX9	pDCs	cytoplasm	TNF-α	coprecipitation of DHX9 with biotin-CpG-B	Kim et al., 2010
DHX36	pDCs	cytoplasm	IFN-α	coprecipitation of DHX36 with biotin-CpG-A	Kim et al., 2010
DDX41	DCs	cytoplasm	IFN-α, β	coprecipitation of dsDNA with HA-tagged DDX41	Zhang et al., 2011b
DDX60	HeLa cells	cytoplasm	IFN-β, CXCL10	dsDNA-dependent shift of purified His-tagged DDX60 migration in gel shift assay	Miyashita et al., 2011
cGAS	L929, THP-1, HEK293	cytoplasm	IFN-β	precipitation of GST-cGAS with biotinylated DNA	Sun et al., 2013
MRE11	MEFs, DCs	cytoplasm	IFN-β, CXCL10, IL-6	precipitation of MRE11 by streptavidin beads in lysates from cells transfected with biotin-dsDNA	Kondo et al., 2013

the CTD structures solved lacked the CTT known to be essential for TBK1 activation, means that unfortunately we still lack insight into how STING gets "activated" to move from the ER and engage with TBK1. One model first proposed by Yin et al. (2012) and recently reviewed by Burdette and Vance (2013) is that STING exists as a constitutive dimer in an autoinhibited state and that CDN binding, or activation of STING by upstream DNA sensors, relieves this autoinhibition to make the CTT available to engage with TBK1. Furthermore, STING might direct TBK1 to do more than just phosphorylate IRF3, because Chen et al. (2011) demonstrated that STING also controls a novel antiviral pathway whereby viruses or cytosolic nucleic acids stimulate STING to recruit STAT6 to the ER for subsequent phosphorylation by TBK1, leading to induction of STAT6-dependent antiviral genes. In fact, because it is currently unknown how or whether STING also controls NF-κB activation in response to cytosolic DNA, it is feasible that STING also directs TBK1 to phosphorylate that transcription factor.

Role for PYHIN Family Proteins in DNA Sensing

Because STING does not directly bind to and detect dsDNA, coupled with the fact that DAI and Pol III are unable to account for all or many of the known cases of dsDNA-induced IFN, proteins acting upstream of STING to detect dsDNA clearly exist. Some PYHIN proteins have been found to fulfill this role. Proteins of the PYHIN family have an amino-terminal pyrin domain, capable of protein:protein interactions, and one or two carboxy-terminal HIN domains, capable of DNA binding. PYHIN proteins have been described to be involved in cell proliferation, survival, and differentiation (Mondini et al., 2010). The human and murine genomes encode 4 and 14 PYHIN proteins, respectively (Cridland et al., 2012). Two human PYHIN proteins, absent in melanoma (AIM2) and IFN-γ-inducible (IFI16) have been demonstrated to be essential for distinct DNA-activated innate responses and have been proposed to be DNA sensors (Hornung et al., 2009; Fernandes-Alnemri et al., 2009; Bürckstümmer et al., 2009; Roberts et al., 2009; Unterholzner et al., 2010). AIM2 localizes to the cytoplasm and binds DNA as evidenced by affinity purification of Myc-tagged AIM2 with dsDNA-coupled beads and direct interaction between rAIM2 and dsDNA in vitro (Hornung et al., 2009). AIM2 is essential for interleukin-1β (IL-1β) production in response to dsDNA including poly(dA:dT) and vaccinia virus DNA and works by assembling an inflammasome with ASC and caspase 1 (Hornung et al., 2009; Bürckstümmer et al.,

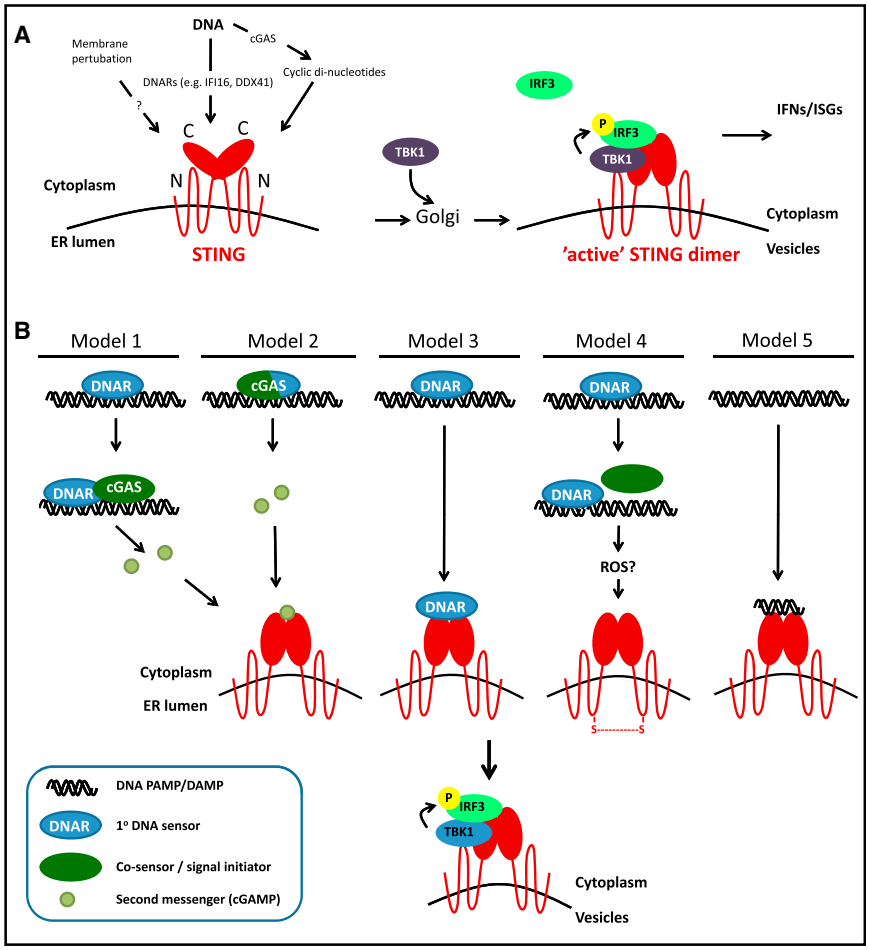

Figure 3. Models for STING Activation

(A) STING resides in the ER either as a monomer or more probably as a dimer (shown) in an auto-inhibited state and is "activated" by intracellular DNA, CDNs, and membrane perturbation. This leads to formation of an active STING dimer and mobilization to perinuclear vesicular structures where the C-terminal domain of STING serves as a platform for TBK1-mediated phosphorylation of IRF3.

(B) Based on the current literature, several models for STING "activation" are possible. (1) DNA is sensed by a DNA sensor that initiates downstream signaling involving production of a second messenger (cGAMP) that binds to STING, causing a conformational change essential for STING to recruit TBK1. (2) cGAS is the initial sensor of DNA and is activated by DNA binding to produce cGAMP, thereby triggering STING activation as in (1). (3) DNA sensors directly interact with STING upon DNA binding and thereby stimulate the conformational change required for STING to recruit TBK1. (4) STING "activation" involves other mechanisms such as redox-regulated covalent linkage of the monomers (Jin et al., 2011). (5) STING binds DNA directly and stimulates downstream signaling (Abe et al., 2013).

2009; Fernandes-Alnemri et al., 2009; Roberts et al., 2009). We showed that human IFI16 binds dsDNA and induces STING-dependent IFN-β responses (Unterholzner et al., 2010). IFI16 was found to colocalize with HSV-1 and HCMV genomic DNA in the cytoplasm in primary human macrophages and to be essential for induction of IFN responses in these cells (Horan et al., 2013). The murine PYHIN protein p204, which has a similar domain organization as human IFI16, was found to be essential for DNA and HSV-1-induced transcription factor activation and IFN-β expression in a mouse macrophage cell line (Unterholzner et al., 2010), even though those cells expressed many other mouse PYHIN proteins, which may indicate that p204 is not redundant with other mouse family members in sensing DNA in myeloid cells.

The structure of PYHIN proteins, with their clearly defined ligand-binding HIN domain and protein-protein interaction signaling domain (pyrin), is consistent with their proposed role as DNA PRRs, and as such AIM2, IFI16, and p204 form a new family of PRRs termed AIM2-like receptors (ALRs). The structure of the AIM2 HIN domain and one of the IFI16 HIN domains, in complex with dsDNA, has now been solved (Jin et al., 2012). This reveals the molecular basis for sequence-independent sensing of dsDNA by the innate immune system, because all the contacts between the HIN domain and the dsDNA are with the DNA phosphate backbone and not with individual nucleotides. This work also suggested a model as to how ALRs are activated by DNA, which involves displacement of an autorepressed pyrin domain from the HIN domain by DNA (Jin et al., 2012). AIM2 would then engage ASC via a pyrin:pyrin homotypic interaction. It is unclear how the pyrin domain of IFI16 contributes to signaling to STING and subsequent IFN induction. To date, pyrin domains have been found to interact only with other pyrin domains, such as in the case of AIM2 and ASC (Park, 2012). IFI16, however, is not widely reported to activate the inflammasome, except in the case of herpesvirus-stimulated IL-1β production after nuclear sensing of DNA (Kerur et al., 2011). In fact, the pyrin domain of IFI16 and other PYHIN proteins seems structurally distinct from AIM2 (Park, 2012) and therefore probably recruits yet-to-be-identified signaling proteins that may have a role upstream of STING activation.

DExD/H-Box Helicases Regulate Intracellular DNA Sensing

The DExD/H-box helicases (DDX) protein family comprises RNA and DNA helicases containing a DExD/H-box domain. DDX proteins have been implicated in the regulation of gene induction at multiple points including signal transduction pathways, gene promoters, mRNA splicing, and translational regulation. Several DDX proteins have been implicated in innate immunity working as RNA sensors (RIG-I and MDA5), signaling molecules (DDX3), and also DNA sensors (Yoneyama et al., 2004; Schröder et al., 2008; Zhang et al., 2011b; Kim et al., 2010). Liu and associates reported that DDX41 can interact with synthetic dsDNA through the DEAD domain in vitro and also showed that DDX41 was required for DNA-dependent induction of type I IFN in myeloid

DCs through a pathway dependent on STING and TBK1 (Zhang et al., 2011b). Interestingly, the authors found that in a cell type with limited basal IFI16 expression, DDX41 seemed to be the initial sensor of cytoplasmic DNA, inducing IFN and subsequent IFI16 expression, the latter serving as an amplifier of innate responses (Zhang et al., 2011b). Thus, data from studies involving IFI16 and DDX41 suggest that the pattern of DNA sensor expression in cells may define which sensor mediates the innate response to intracellular DNA. In addition to the proposed role for DDX41 as a DNA sensor, it was recently reported that DDX41, like STING, directly binds CDNs and that CDN-induced IFN was DDX41 dependent (Parvatiyar et al., 2012). The relative role of STING versus DDX41 in sensing CDNs is not yet resolved, but it is possible that DDX41 plays a role as an essential signaling molecule for STING-dependent DNA and CDN responses, rather than as an initial sensor of DNA and CDNs. Further evidence for a central role for DDX41 in DNA-induced STING-dependent responses came from a recent paper from Zhang et al. (2013) demonstrating that the E3 ubiquitin ligase TRIM21 was a negative regulator of DNA responses both in vitro and in vivo and that TRIM21 targeted DDX41 for degradation.

In addition to DDX41, three other DExD/H-box helicases have been ascribed roles in innate DNA sensing. In a screen for cytosolic DNA-interacting proteins in pDCs, Kim et al. (2010) identified DHX9 and DHX36, which bind CpG DNA and interact with MyD88. In vitro infection with HSV-1 evoked MyD88-dependent TNF-α and IFN-α responses, which were partly dependent on DHX9 and DHX36, respectively. Recent studies have demonstrated that DHX9 interacts not only with DNA but also with RNA, which induces MAVS-dependent IFN and cytokine expression in myeloid DCs (Zhang et al., 2011b, 2011c). This raises important questions about the emerging roles of the DDX superfamily in intracellular sensing of nucleic acids (Zhang et al., 2011b, 2011c; Kim et al., 2010). For instance, as discussed above for DDX41, are DHX9 and DHX36 actual DNA sensors or rather essential downstream signaling molecules in the nucleic acid recognition pathways? The RNA PRRs RIG-I and MDA5 have a defined signaling domain (CARD) as well as a nucleic acid binding DDX domain, and crystal structures for both receptors have revealed how they engage RNA and subsequently signal (Kowalinski et al., 2011; Luo et al., 2011; Wu et al., 2013a). Likewise, for the PYHIN proteins, cooperative binding of DNA to the HIN domains has been demonstrated (Unterholzner et al., 2010), and the structural determination of the HIN:DNA complex suggests a mechanism whereby the signaling pyrin domains are mobilized (Jin et al., 2012). In contrast, it is currently unclear how DDX41 might bind nucleic acid ligands and then transduce signals, especially because all of the interactions with nucleic acid and downstream proteins observed to date are shown to involve the DEAD domain (Zhang et al., 2011b; Parvatiyar et al., 2012). Therefore, more work is needed to specifically dissect the roles in these pathways, in particular whether DDX41 is a DNA sensor or rather an essential signaling molecule upstream of STING.

Cyclic-di-GMP-AMP Is a Second Messenger in DNA Signaling to STING

In an exciting new development that yields further insight into how STING is activated by dsDNA sensing, two papers from the group of Z. Chen demonstrated that stimulation of cells with cytosolic DNA induced synthesis of cyclic-di-GMP-AMP (cGAMP) from ATP and GTP by a cyclase enzyme called cGAMP synthetase (cGAS), leading to STING-dependent induction of IFN (Sun et al., 2013; Wu et al., 2013b). cGAMP, whose structure resembles CDN molecules, directly bound to STING, and as such cGAMP may represent the endogenous STING activator that bacterial CDNs "mimic" to enable IFN induction. This new cGAS-cGAMP second messenger system is reminiscent of the classic cAMP second messenger pathway whereby the enzyme adenylate cyclase generates cAMP from ATP in response to G protein-coupled receptors. GST-cGAS fusion proteins were demonstrated to interact directly with dsDNA, primarily through an amino-terminal domain, and the interaction led to synthesis of cGAMP. The ability of IFI16 and DDX41 to stimulate cGAS activity is now urgently needed to be tested in order to ascertain whether the cGAS system is utilized by proposed upstream DNA sensors (Figure 3). How broadly the cGAS system operates in different cell types is currently unclear. Despite this, cGAS was demonstrated to be essential for induction of IFN-β expression by DNA viruses in a mouse fibroblast and human monocytic cell line (Sun et al., 2013; Wu et al., 2013b).

Immune Sensing of DNA in the Nucleus: A Link with the DNA Damage Response?

The studies described above demonstrate significant progress in understanding the biochemistry of DNA sensing and in identifying the signaling proteins involved. In parallel, there has been strong interest in elucidating the cell biology of DNA detection. An early dogma was that the nucleus is immune privileged for DNA detection and that the presence of DNA outside of the nucleus constitutes the key signal for immune activation, but this might not be the case. Interestingly, IFI16 is predominantly localized in the nucleus, and it was speculated early that IFI16 might also acts as a PRR in the nucleus (Goubau et al., 2010). A report by Kerur et al. (2011) subsequently demonstrated IFI16 to sense Kaposi's sarcoma-associated herpesvirus DNA in the nucleus of endothelial cells leading to activation of an IFI16-ASC-Caspase 1 inflammasome in the cytosol. This was followed by a report showing that IFI16 senses HSV-1 DNA in the nucleus of permissive cells (Li et al., 2012; Orzalli et al., 2012). Given the proposed ability of IFI16 to act as a DNA sensor both in the cytosol and the nucleus, information on what determines the subcellular localization of IFI16 is important. Li et al. (2012) identified acetylation of the nuclear localization signal in IFI16 as a mechanism to promote cytoplasmic localization, and it will be interesting to learn more about how cells and microbes control this process.

These findings challenge the model that location is the only factor determining whether DNA can activate innate immune responses and demonstrate the need to better understand how foreign DNA in the nucleus is distinguished from self-DNA. Alternatively, it may be that any nuclear DNA not normally complexed with histones in chromatin is immune stimulatory. In that regard it is very interesting to note that the DNA damage response (DDR), which is activated by abnormalities in DNA such as double-stranded breaks, has been shown to activate NF-κB and IRFs and to induce IFN (Brzostek-Racine et al., 2011). The authors of that study noted that both RNA and DNA viruses in the nucleus can generate breaks in DNA during integration and lytic

replication that activates the DDR, suggesting that the DDR and viral detection in the nucleus may be integrated responses. Intriguingly, prior to its identification as an innate DNA sensor, IFI16 was previously know to associate with BRCA1 at genomic sites of DNA damage (Aglipay et al., 2003).

Consistent with the proposed link between the DDR and immune stimulation by DNA, a central kinase in the DDR, DNA-dependent protein kinase (DNA-PK), has also been implicated as an innate immune DNA sensor (Table 1 and Figure 2). DNA-PK is a heterotrimeric protein complex consisting of three subunits, DNA-PKcs (DNA-PK catalytic subunit), Ku70, and Ku80. The latter two proteins form a heterodimeric complex and are involved in detection of dsDNA breaks during DNA damage, leading to rapid activation of the catalytic DNA-PKcs subunit and initiation of nonhomologous end joining in the DDR (Ciccia and Elledge, 2010). Two publications now demonstrate a role for DNA-PK in DNA sensing in HEK293T cells and murine fibroblasts. Zhang et al. (2011a) first demonstrated that knockdown of Ku70 suppressed IFN-λ1 induction in response to linear plasmid DNA in HEK293 cells, with no effect on the type I IFN (IFN-α/β) response. Ferguson et al. (2012) reported that DNA-PK detects DNA in the cytosol of fibroblasts in particular and induces expression of IFN-β and other genes in response to DNA and to vaccinia virus. The published data showed that long DNA induced more IFN-λ1 than short DNAs and that linear DNA was more stimulatory than circular DNA (Zhang et al., 2011a). Because Ku70 and Ku80 detect DNA ends during the DDR, DNA-PK may detect free DNA ends and act in concert with other DNA sensors recognizing dsDNA to mount IFN responses. Most recently, a further link between the DDR and immune responses to DNA has emerged since the DNA damage sensor MRE11 has been shown to be involved in STING-dependent responses cytosolic to dsDNA but not to DNA virus (Kondo et al., 2013).

Role for DNA Sensing in Antimicrobial Immunity

The functional consequence of innate immune recognition of DNA and whether it plays a beneficial or pathological role depends on the biological context. The understanding of DNA sensing in host defense and immunopathology has not advanced to the same extent as has the biochemical and cell biological research in this field. This is due to the lack of data from gene-modified mice lacking key components in the DNA-sensing pathway, notwithstanding the availability of the STING knockout mouse, which has been very informative in demonstrating in vivo roles for STING. For other signaling proteins implicated "upstream" of STING, much of the current knowledge is based on RNA interference experiments. By far the most-studied microorganisms with respect to DNA sensing are herpesviruses, and in particular HSV (Paludan et al., 2011). All of the proposed DNA sensors have been demonstrated to play a role in innate sensing of herpesviruses, in the first instance TLR9 and more recently the cytosolic DNA sensors. For example, human primary monocyte-derived macrophages produce IFN-β in an IFI16-dependent manner after HSV-1 infection (Horan et al., 2013), and primary murine DCs evoke type I IFN responses via DDX41 in response to the same virus (Zhang et al., 2011b). Interestingly, DAI/ZBP1, originally described to drive IRF3 activation, has subsequently been reported to play a key role in necroptosis after infection with MCMV (Upton et al., 2012). Because most if not all of these DNA-stimulated functions rely on STING, it is no surprise that STING-deficient mice are highly susceptible to HSV-1 infection (Ishikawa et al., 2009). Based on the work conducted with herpesviruses, it seems possible that some degree of cell type specificity applies to the function of DNA sensors in primary cells.

HIV has a replication cycle that involves RNA, ssDNA, RNA:DNA hybrids, and dsDNA, and therefore there is potential for a role for several classes of nucleic acid sensors of RNA and DNA in HIV recognition (Solis et al., 2011; Berg et al., 2012; Doitsh et al., 2010; Yan et al., 2010). One study focusing on IFN responses to HIV found that elimination of expression of TREX1 augmented HIV-induced type I IFN responses (Yan et al., 2010). TREX1 is a 3′-5′ exonuclease and the IFN response to ssDNA was more sensitive to the presence of TREX1 than was the IFN response induced by dsDNA. This suggests that the HIV induced IFN response involves a PRR sensing ssDNA. The sensors driving this response have not been identified, but similar to dsDNA responses they were found to signal through the STING-TBK1-IRF3 pathway (Yan et al., 2010).

The IFN response to some bacteria can also be driven by intracellular DNA (Stetson and Medzhitov, 2006), and DDX41 and p204 have been reported to be essential for optimal IFN-β responses to *Listeria monocytogenesis* and *Mycobacterium tuberculosis*, respectively (Zhang et al., 2011b; Manzanillo et al., 2012). However, given the ability of bacteria-derived CDNs to directly stimulate IFN induction via DDX41 and STING (Parvatiyar et al., 2012; Burdette et al., 2011), it may be difficult to assess the relative contribution of bacterial DNA versus CDNs to induction of IFN expression during some bacterial infections. Interestingly, the cytosolic DNA-sensing pathway is also involved in recognition of extracellular bacteria (Charrel-Dennis et al., 2008; Koppe et al., 2012). *Streptococcus pneumonia* stimulated the STING-IFN pathway in macrophages through a mechanism dependent on the pore-forming toxin pneumolysin (Koppe et al., 2012). Beyond IFN induction, the DNA-sensing signaling machinery has been reported to stimulate autophagy during *M. tuberculosis* infection, which was essential for optimal antimicrobial defense and was dependent on STING and TBK1 (Rasmussen et al., 2011; Watson et al., 2012). As noted earlier, the work on *M. tuberculosis* revealed that the bacteria were actually exploiting DNA-activated responses, as demonstrated by IRF3-dependent establishment of long-term infection (Manzanillo et al., 2012).

Some parasites pass through an intracellular stage during their life cycle and hence might be recognized by the innate immune system via DNA sensors. TLR9 has been convincingly demonstrated to be able to detect *Toxoplasma gondii*, *Plasmodium falciparum*, and *Trypanosoma cruzi* (Minns et al., 2006; Pichyangkul et al., 2004; Bafica et al., 2006). Patients with malaria exhibit an elevated type I IFN response in the blood, and murine studies indicate a role for IFN in the pathogenesis of malaria (Pichyangkul et al., 2004; Franklin et al., 2009; Sharma et al., 2011). The *P. falciparum* genome, which has an A/T content of about 80%, potently stimulates STING-dependent type I IFN responses through an unidentified DNA sensor (Sharma et al., 2011). Interestingly, the immunostimulatory DNA was not dsDNA per se, but rather hairpin loop DNA, suggesting that innate sensing of DNA is

not limited to dsDNA for important pathogens such as *P. falciparum* and HIV.

Role for DNA Sensing in Autoimmunity

In addition to microbial DNA, self-DNA also has the potential to trigger innate immune responses, and both type I IFNs and TLR9 have established roles in mouse models of autoimmunity and in human patients with autoimmune disease (Leadbetter et al., 2002; Bennett et al., 2003; Baechler et al., 2003; Agrawal et al., 2009; Christensen et al., 2006; Yu et al., 2006; Santiago-Raber et al., 2010). The cytosolic DNA-sensing pathways are also likely to play a role in autoimmunity given the link between TREX1 and some inflammatory diseases: lack of TREX1 causes cytosolic accumulation of DNA originating from endogenous retroelements and replication by products leading to the development of DNA-driven, IFN-dependent autoimmune diseases (Stetson et al., 2008; Gall et al., 2012). Interestingly, in humans, TREX1 mutants are associated with the immune-mediated neurodevelopmental disorder AGS (Crow et al., 2006). It will be interesting to learn which DNA sensors are involved in detection of DNA in TREX1-insufficient individuals and also to gain a full understanding of how cells normally keep the cytoplasm clear of DNA.

Apart from TREX1 containment of endogenous retroelements, failure to clear DNA from apoptosed dead cells also seems to trigger autoimmunity driven by self DNA. Mice lacking DNase II are unable to efficiently eliminate self DNA through the lysosomal pathway and leakage of DNA to the cytoplasm occurs (Yoshida et al., 2005; Kawane et al., 2001). DNase II-deficient mice die during embryonic development at least partly due to anemia (Kawane et al., 2001), which is rescued in mice also deficient in the type I IFN receptor (Yoshida et al., 2005). However, these mice develop polyarthritis because of production of inflammatory cytokines such as TNF-α (Yoshida et al., 2005). Clear evidence that the cytosolic DNA-sensing pathway is involved in the pathology evoked by accumulating DNA resulting from defects in lysosomal functions comes from a report that mice deficient in both DNase II and STING are rescued from both embryonic lethality and polyarthritis (Ahn et al., 2012).

All together, although the field of DNA sensing is relatively new, there is already ample evidence for the importance of this machinery in host defense to infections and development of autoimmune diseases. Development of more gene-modified mouse strains will allow further progress in this area. Finally, although not discussed in this review, it has also been reported that the cytosolic DNA-sensing machinery stimulates adaptive immune responses and hence at least partially accounts for the known adjuvancy properties of DNA (Ishii et al., 2008; Kis-Toth et al., 2011).

Concluding Remarks

This review has illustrated the rapid progress that has been made in understanding and characterizing the innate immune response to cytosolic DNA, a topic that we knew virtually nothing about 6 years ago. Cytosolic signaling pathways activated by DNA have been uncovered, new DNA receptors proposed, and we now know that innate DNA recognition is closely connected to the process of host defense against microbial infection as well as development of some autoimmune diseases. All of this research activity has raised some interesting questions that remain to be answered relating to mechanisms of DNA detection. For example, how do PYHIN and DDX proteins engage the STING-TBK1-IRF3 signaling axis? What is the role of the proposed DNA sensors, and cGAS, in vivo, and is there redundancy or cell specificity between different potential sensing systems? How does nuclear sensing of viral DNA relate to sensing of DNA damage, and how might such signals be propagated to STING in the cytosol? It will also be important to more fully understand the cell biology of DNA sensing, particularly as it relates to STING function, and to determine which chaperone and sorting adaptor proteins are required to direct correct signaling responses (Kagan, 2012).

Research on innate immune DNA sensing has also provoked broader questions related to immunology, such as does DNA sensing distinguish self from non-self at all, or is it primarily danger (such as mislocalized DNA or DNA damage) that is being sensed to drive IFN induction? Another key question is how accurately the mouse system recapitulates DNA responses in humans, as we seek to apply this new knowledge to both pathogen-driven and autoimmune human disease. Although so far mouse and human STING appear to function similarly, the fact the humans have just 4 PYHIN proteins whereas mice have 14 provides an interesting snap-shot into potential difficulties in modeling DNA sensing in mice. It is likely that future discoveries on DNA-stimulated innate and adaptive immune responses will unveil further novel signaling mechanisms and also continue to reveal key roles of this part of the immune system in infections and inflammatory diseases.

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